

GENETIC RECOMBINATIONS LEADING TO PRODUCTION OF ACTIVE BACTERIOPHAGE FROM ULTRAVIOLET INACTIVATED BACTERIOPHAGE PARTICLES¹

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THE potentialities of bacteriophage genetics have been revealed by the discovery of genetic recombinations among related phage particles infecting the same bacterial cell (DELBRÜCK and BAILEY 1946). The complexities of these genetic systems have been further illustrated by the work of HERSHEY and ROTMAN (1948) on a number of different genetic determinants involved in the determination of the alternative phenotypes r^+ and r in phage *T2H*. A different approach to the genetic mechanisms of bacteriophages originated from the chance observation by DELBRÜCK and BAILEY that, after exposure to ultraviolet light, some phages gave variable plaque counts, depending on the relative concentrations of phage lysates and host cells at the time of assay. In investigating this phenomenon, one of us (LURIA 1947) discovered a mechanism of phage reactivation by interaction among inactive particles in the course of intracellular growth. The detailed investigation of this phenomenon has indicated new possibilities for a quantitative analysis of the genetic structure of these viruses and has suggested a possible mechanism for their reproduction.

A preliminary discussion of some of the results reported in this paper has appeared (LURIA 1947); their implications for a number of problems have been discussed in a forthcoming publication (LURIA 1948). The present article is intended to present the results in detail, and, by describing techniques and methods of analysis, to serve as a background for future publications on this topic.

The analysis of the results presented in the following pages is based on the hypothesis that inactivation of bacteriophage particles by ultraviolet light is due to production of discrete alterations in individual portions of genetic material. Although the internal evidence in support of this hypothesis, as presented in this paper, is quite satisfactory, it must be said that satisfactory external evidence from other lines of attack is not yet available. The conclusions reached in this article must be considered for the time being as working hypotheses for further investigation.

MATERIAL AND GENERAL METHODS

The system of phages *T1-T7*, their r mutants, and their common host *Escherichia coli* strain B have repeatedly been described, as well as the use of

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bacterial mutants resistant to one or more phages as indicators for one phage in the presence of another (see DELBRÜCK 1946). Plate counts for viable bacteria, and plaque counts in agar layer for active phage were used throughout, employing 1.1 percent agar in "Difco" nutrient broth plus 0.5 percent NaCl. All plates were incubated at 37°C. Experimental bacterial cultures in the logarithmic phase of growth were grown with aeration at 37°C from standard inocula.

The phage stocks were lysates in glucose+ammonia (or lactate+ammonia) medium. These media give negligible absorption of the ultraviolet light used in this work. High titer phage lysates (over 1×10^{11} particles per ml) might give some ultraviolet screening effect because of bacterial debris and of phage itself. Whenever possible, therefore, phage was irradiated after a dilution 1:5 or higher in the same medium. The source of ultraviolet was a General Electric Company germicidal bulb, 15 watts, alimented through a stabilizer. At a distance of 50 cm from the center of this bulb, the flux—measured with a Westinghouse SM-200 meter with tantalum phototube WL-775—is about $7 \text{ erg} \times \text{mm}^{-2} \text{ sec}^{-1}$. The beam contains mainly radiation of wavelength 2537 Å. Samples were irradiated in a thin layer (not over 0.4 mm) in open Petri dishes rocked during exposure.

The technique of "one-step growth" experiment in its various forms has been described in detail previously (DELBRÜCK and LURIA 1942).

EXPERIMENTAL

Inactivation and reactivation of bacteriophages

Plaque counts on phage suspensions exposed to ultraviolet for various lengths of time generally give survival ratios whose logarithms are proportional to the dose, that is, to the time of exposure (see LATARJET and WAHL 1945, and figure 1). The logarithmic rate indicates a one-hit mechanism of inactivation (LEA 1947), and we can assume that the hit consists of the successful absorption of one quantum. The probability that one quantum produces inactivation is, however, very small: for phage T2, for example, one inactivating hit is produced by a dose corresponding to almost 10^4 quanta absorbed per particle (M. ZELLE, personal communication). Only one absorption in 10^4 on the average is, therefore, effective, the others probably producing excitations that do not lead to the inactivating effect.

When the average number of effective hits per particle is r , the proportion of active to total phage will be e^{-r} . For $r=1$, $e^{-r}=0.37$; the corresponding dose is the "inactivation dose" in LEA's terminology (1947). If doses are expressed in multiples of the inactivation dose, their values give directly the average number of hits per particle.

Phage particles inactivated by ultraviolet light are adsorbed by bacteria (LURIA and DELBRÜCK 1942). This is detected because adsorption of one particle by a bacterium causes death of the latter. One can, therefore, measure the rate of adsorption of inactive particles from the survival of bacteria in mixtures containing bacteria and irradiated phage in known proportions. If, on the average, x particles are adsorbed per bacterium, a fraction e^{-x} of the

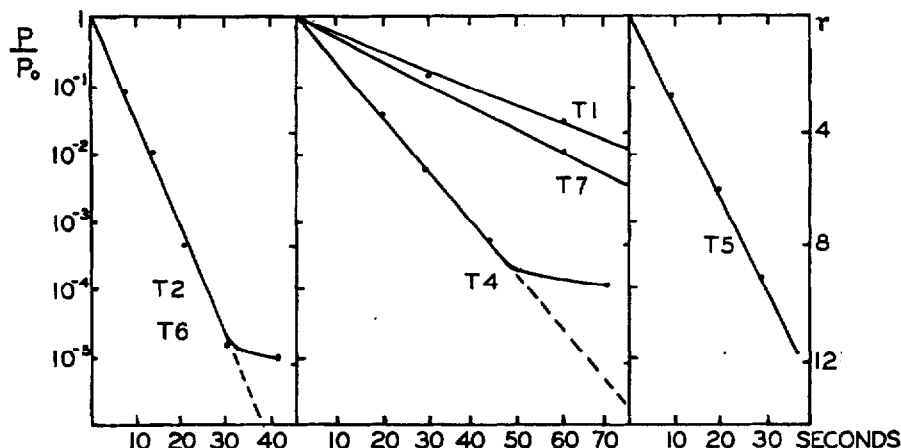


FIGURE 1.—Inactivation of various phages by ultraviolet light. P/P_0 =proportion of active phage particles after irradiation. $r=\ln P_0/P$ =average number of lethal hits per phage particle. The doses are expressed in seconds of exposure. The deviations for high doses in the curves for T_2 , T_4 , and T_6 are due to reactivation occurring on the assay plates (see text). The broken lines represent extrapolations from the logarithmic portions of the curves.

bacteria should receive no particle and survive. By this means, we could establish that for irradiated phages T_2 , T_4 , T_5 , and T_6 , even for high doses, the rate of adsorption is the same as for unirradiated phage. An experiment of this type is shown in table 1. Only for very high doses a slight reduction occurs in the ability of phage to kill bacteria. This reduction is never such as to require important corrections in the analysis presented in later sections.

With phages T_2 , T_4 , T_5 , and T_6 (the "large particle" phages) the plaque counts on irradiated samples are not independent of the mode of assay. They depend on the concentration of the samples when first mixed with bacteria, in a way illustrated in table 2. In these experiments, bacteria were mixed with various concentrations of irradiated phage. Before lysis and phage liberation

TABLE 1

Killing of bacteria by irradiated phage

0.9 ml of a bacterial culture was mixed with 0.1 ml of each of five suspensions of phage T_2 that had received various doses of radiation. After 10 minutes, samples were diluted and plated for viable bacterial count.

EXPERIMENT NO.	PHAGE INPUT, PARTICLES PER ML	BACTERIAL INPUT, CELLS PER ML	DOSE OF RADIATION		SURVIVING BACTERIA PER ML	SURVIVAL INPUT e^{-x}	PHAGE ADSORBED PER BACTERIUM x
			SECONDS	HITS			
				PER PARTICLE			
129	2×10^9	1.15×10^9	0	0	2.3×10^8	0.20	1.60
			60	18	2.4×10^8	0.21	1.56
			70	21	2.2×10^8	0.19	1.66
			80	24	1.6×10^8	0.14	1.96
			100	30	2.7×10^8	0.23	1.47

took place, dilutions were made to bring the total dilution of the irradiated sample to a constant value, and an aliquot plated for plaque count. The plaque counts represent infected bacteria that liberate active phage. Although the total dilution of the irradiated phage on all plates is the same, it is seen that the plaque counts are higher when bacteria have first been placed in contact with a more concentrated phage lysate.

This means that bacteria may produce active phage if they pick up the ir-

TABLE 2

Dependence of plaque counts on irradiated phage T6r on the concentration of the phage sample that is mixed with bacteria

A sample of phage T6r containing 1.5×10^{10} particles/ml was irradiated for 20 seconds. The bacterial suspension (B) contained 2×10^8 cells/ml. Each plate received 0.05 ml of phage dilution and 0.2 ml of suspension (B).

EXPERI- MENT NO.	MIX- TURE NO.	PROCEDURE	DILUTION OF PHAGE WHEN FIRST MIXED WITH (B)	TOTAL DILU- TION FROM THE ORIGINAL PHAGE TO THE SUSPENSION FROM WHICH SAMPLES ARE PLATED	PLAQUE COUNT (SUM OF TWO PLATES)
	1	0.1 ml T6r → 0.9 ml (B); kept 10 min. at 37°C; diluted 1:10 ³ , 0.05 cc plated	1:10	1:10 ⁴	1318
	2	0.1 ml (T6r 1:10) → 0.9 ml (B); kept 10 min. at 37°C; diluted 1:10 ³ , 0.05 ml plated	1:10 ³	1:10 ⁴	474
5	3	0.1 ml (T6r 1:10 ³) → 0.9 ml (B); kept 10 min. at 37°C; diluted 1:10, 0.05 ml plated	1:10 ⁴	1:10 ⁴	250
	4	0.05 ml (T6r 1:10 ⁴) plated	Less than 1:10 ⁵ (on plate)	1:10 ⁴	57

radiated particles from a concentrated phage suspension, but not from a dilute one. The immediate explanation is that from a concentrated lysate the bacteria receive some other "factor," which, inside the bacterium, somehow *reactivates* an "inactive" particle and which is not present in dilute lysates. An "inactive" particle can be defined as one that has lost the ability to initiate production of active phage unless adsorbed by a bacterium together with the unknown "factor."

Reactivation still occurs after storage of irradiated phage for weeks in an ice-box. Reactivation gives rise to fully active phage particles. This can be proved either by sampling phage from the plaques or by letting the bacteria, in which reactivation occurs, lyse in liquid and then testing the lysate for active particles.

The occurrence of reactivation for certain phages accounts for deviations from the logarithmic inactivation rate found for these same phages (see figure 1). As the dose of radiation increases beyond a certain point, the survival, as determined by plaque count, appears to diminish less rapidly. This is due to an unavoidable partial reactivation. In a phage titration, we mix approximately 5×10^7 bacteria with an amount of phage suspension such as to give approximately 100 plaques, and pour the mixture on an agar plate. On the one hand, if we are dealing with a fully active sample, the bacteria only come in contact with 100 active particles. On the other hand, in assaying an irradiated suspension containing, for example, one active particle in 10^6 , we expose the bacteria to 10^8 inactive particles plus a correspondingly large amount of other lysate constituents, besides the residual 100 active particles. Conditions permitting reactivation, therefore, obtain in these plating mixtures, and reactivation disturbs and often completely obscures the count of the residual active phage. For this reason, the survival of fully active phage for high doses must be obtained by extrapolation from the logarithmic part of the curve, a necessarily inefficient procedure. If deviations in the survival rate for high doses occurred, this extrapolation would not be justified. One possible cause of error—screening of some phage particles from radiation by components of the lysate itself—was excluded by irradiating concentrated phage *T6* mixed with phage *T1*, and testing for the inactivation rate of the latter, which is not disturbed by reactivation phenomena. The inactivation rate of *T1* remains the same as in the absence of *T6* up to doses that correspond to 100 hits per particle of phage *T6*.

Identification of the reactivating factor

What is the "factor" present in irradiated stocks of phages *T2*, *T4*, *T5*, or *T6*, which, if acting on bacteria that have adsorbed inactive phage particles, allows production of active phage? Since phage stocks are lysates produced by lysis of the common host *E. coli* B, the factor might be either of phage or of bacterial origin.

The factor was identified as being phage itself, inactive or active, in that *reactivation occurs in bacteria that adsorb either more than one inactive particle of a given phage, or one inactive particle of one phage plus some active or inactive particles of a related phage*. The evidence for this conclusion, which is illustrated in part by the data in table 3, can be summarized as follows.

(a) Addition of an excess of supernatant from a heavy bacterial culture to a mixture of dilute irradiated phage plus bacteria gives no increase in plaque count. The factor in the lysates is not a normal bacterial secretion.

(b) Concentrated lysates of phages *T1*, *T5*, or *T7* added to a mixture of bacteria and dilute irradiated *T2* (or *T4*, or *T6*) do not cause reactivation. No heterologous lysate causes reactivation of *T5*. The test for cross-reactivation is done by plating the mixtures, before lysis, with bacterial indicator strains sensitive to the phage whose reactivation is tested, but not to the others. Since all phage stocks are lysates of common host cells, the factor is not an unspecific bacterial product liberated upon lysis.

TABLE 3

Reactivation of phage T2 under various conditions

Phage *T2*, containing 2×10^{10} particles per ml, was irradiated for 35 seconds. Phages *T6* and *T4*, containing 4×10^{10} particles per ml, were irradiated for 30 seconds. Phages *T1* and *T5* contained 3×10^{10} particles per ml. The bacterial suspensions (B) and (B/6) contained 10^9 cells per ml.

MIXTURE NO.	CONTENTS	AFTER 10 MINUTES	PLAQUE COUNT (SUM OF TWO PLATES)	REACTIVATION
1	0.1 ml Phage <i>T2</i> dil. 1:500+1.9 ml (B)	0.05 ml plated with B	22	—
2	0.1 ml Phage <i>T2</i> dil. 1:500+1.9 ml (B/6)	0.05 ml. plated with B/6	20	—
3	0.1 ml Phage <i>T2</i> pure +1.9 ml (B)	dil. 1:500, 0.05 ml plated with B	4000	+
4	0.1 ml Phage <i>T2</i> pure +1.9 ml (B/6)	dil. 1:500, 0.05 ml plated with B/6	3000	+
5	0.1 ml Phage <i>T2</i> . dil 1:500+0.1 ml irradiat. <i>T6</i> +1.8 ml (B)	0.05 ml plated with B/6	670	+
6	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml unirrad. <i>T6</i> +1.8 ml (B)	0.05 ml plated with B/6	190	+
7	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml irradiat. <i>T4</i> +1.8 ml (B)	0.05 ml plated with B/4	661	+
8	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml irradiat. <i>T6</i> +1.8 ml (B/6)	0.05 ml plated with B/6	10	—
9	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml unirrad. <i>T6</i> +1.8 ml (B/6)	0.05 ml plated with B/6	12	—
10	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml irradiat. <i>T4</i> +1.8 ml (B/6)	0.05 ml plated with B/4	883	+
11	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml unirrad. <i>T1</i> +1.8 ml (B)	0.05 ml plated with B/1, 5	18	—
12	0.1 ml Phage <i>T2</i> dil. 1:500+0.1 ml unirrad. <i>T5</i> +1.8 ml (B)	0.05 ml plated with B/1, 5	17	—

(c) Concentrated lysates of phages *T2* (or *T4*, or *T6*), whether fully active or irradiated, can reactivate irradiated particles of any other *T-even* phage. These phages are morphologically, serologically, and probably genetically related, whereas *T5* belongs to a fully separate group (DELBRÜCK 1946). The "factor" in the lysates appears to carry the same pattern of relatedness. It is not produced by irradiation, since its presence can be proved in unirradiated lysates by the technique of cross-reactivation.

(d) Reactivation is independent of contact between phages prior to infection of the host: a mixture of bacteria and phages gives the same amount of reactivation independently of how long the phages have been together before adding the bacteria.

(e) Phage *T4r* purified by fast centrifugation (kindly supplied by DR. T. F. ANDERSON) shows both self-reactivation as a function of concentration in the mixtures, and ability to reactivate phages *T2* or *T6*. The factor, therefore, is present in such a purified phage suspension.

(f) Reactivation of phage *T2* by lysates of *T6*, for example, only occurs in presence of bacteria capable of adsorbing both phages. Inactive phage *T2* in presence of bacteria *B/6*, by which it is adsorbed, is not reactivated by *T6*, which is not adsorbed, but is reactivated by *T4*, which is adsorbed. This proves

that the "factor" has the same host specificity as the phage in whose lysate it is found.

The last point, particularly, was considered crucial in showing that production of active phage from an inactive particle was actually due to infection of the same bacterial cell with other particles, either of the same or of a different but genetically related phage, active or inactive. Further confirmation came from the experiments discussed below, which showed that in a mixture of inactive phage and bacteria the number of bacteria yielding active phage is never greater than the number of bacteria that adsorb two or more inactive particles, and in some cases actually equals it. The same holds true for reactivation of an inactive phage, for instance *T2*, by a related one, for instance *T4*; the number of bacteria liberating active *T2* is lower than—or equal to—the number of bacteria receiving at least one particle of each phage. The analysis of cross-reactivation between these different wild-type phages will not be discussed further in this paper, but will form the subject of a future publication.²

(g) All bacteria which, after infection with inactive particles, do not liberate active phage also fail to lyse. This was proved by mixing bacteria and inactive phage under conditions in which some reactivation occurs, plating a sample of the mixture for plaque count, and another sample for direct microscopic observation of lysis on agar. The results of such experiments proved that the fraction of bacteria that are lysed is the same as the fraction of bacteria that liberate active phage. The other infected bacteria fail to grow and divide, and can be seen still apparently unchanged 24 hours later.

Reactivation and genetic transfer

The limitation of cross-reactivation to the *T-even* phages immediately brought out a similarity between this phenomenon and that of genetic transfer described by DELBRÜCK and BAILEY (1946). In the latter case, bacteria simultaneously infected with the phages *T2r*⁺ and *T4r*—the *r* character being the result of mutation from the wild-type, which can be designated as *r*⁺—liberate a mixture of particles of the four types, *T2r*⁺, *T2r*, *T4r*⁺, and *T4r*, among which the second and third represent new types. These must owe their origin to some sort of recombination involving the genetic determinants for the alternative *r*⁺ and *r* phenotypes. Evidence for the discrete nature of these determinants has since been reported by HERSHEY and ROTMAN (1948).

We assumed then, as a working hypothesis for the analysis of the reactivation phenomenon, that inactivation by ultraviolet light resulted from "lethal mutations" in a number of discrete genetic determinants among inactive particles in the same bacterium to reconstitute fully active particles. This hypothesis can be formulated quantitatively in terms of measurable

² Cross-reactivation between *T-even* phages has the limitation that the individual phages are distinguishable only by test of differential properties such as ability to grow on different hosts and rate of inactivation by different antisera. Cross-reactivation can only be defined as the production, upon mixed infection, of active particles having the distinctive properties of an inactive parent particle.

quantities by making a number of simple assumptions. We shall first develop this simple theory and then describe the experiments by means of which it was tested.

Theory

We shall assume that in each particle of a given phage there exist n "units" (or "loci") each capable of undergoing a lethal mutation when exposed to ultraviolet light. Since one effective hit is sufficient to inactivate a phage particle, a lethal mutation can be defined as an effective hit, that is, as an alteration of one unit which makes the phage particle unable to initiate by itself the production of active phage in a bacterium. A particle may undergo more than one lethal mutation, and mutations will be distributed at random and independently among the different units, the distribution depending only on the sensitivity of each unit.

We shall now make the assumption that the sensitivity of all units is the same, and show later that this assumption, if incorrect, only requires a numerical correction which does not invalidate the applicability of the theory.

Our next assumption is that *active phage cannot be produced in a bacterium unless the infecting particle or particles, taken as a group, contain at least one copy of each unit in non-lethal form*. This assumption is an essential feature of the theory, and corresponds to treating each unit as a *discrete, material, independent hereditary unit* endowed with genetic continuity and individuality. An inactive unit cannot be replaced by copies of different units. This assumption implies that production of active units cannot result from the cooperation of two or more lethal units, but only from actual reproduction of active units.

When a population consisting of M phage particles is irradiated with a given dose, there will be produced in each particle, on the average, r lethal mutations, or a total of $M \times r$ mutations in the whole population. Since M particles contain $M \times n$ units, each unit will receive on the average $Mr/Mn = r/n$ lethal mutations.

A given unit, taken at random, will have a probability $e^{-r/n}$ of not having a lethal mutation, and a probability $(1 - e^{-r/n})$ of having at least one.

We ask next: what is the probability that each of the n units is present in at least one non-lethal copy in a group of k particles that enter a bacterium? According to our assumptions, this probability should represent an upper limit for the probability that a bacterium produces active phage.

If a bacterium is infected by k particles, the probability that a given unit is lethal in all of them is: $(1 - e^{-r/n})^k$, and the probability that it is non-lethal in at least one of them is: $1 - (1 - e^{-r/n})^k$.

The probability that at least one non-lethal copy of each of the n units is present in the k particles is the product of the probabilities referred to the individual units. Since we have assumed equal sensitivity for all units—that is, r/n constant for all units for each value of r —the product will be

$$[1 - (1 - e^{-r/n})^k]^n. \quad (1)$$

The expression (1) represents the probability that a bacterium infected by k particles receives at least one full non-lethal complement of the n units.

In a mixture of phage with bacteria, however, there is a distribution of the number of phage particles infecting individual bacteria. With relatively good approximation—see Appendix—this distribution can be considered as a Poisson distribution. If x is the average number of particles adsorbed per bacterium, the fraction of bacteria with k particles is: $x^k e^{-x}/k!$

The fraction of bacteria receiving k particles which carry a full complement of non lethal units is then:

$$\frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \quad (2)$$

Finally, the fraction of the total bacterial population which receives all units in a non-lethal form is the sum of the expression (2) for all possible values of k :

$$Z = \sum_{k=0}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \quad (3)$$

This expression embodies the following consequences of our hypothesis:

(a) No full complement of active units can be present in uninfected bacteria ($k=0$).

(b) Of the bacteria with one phage particle ($k=1$), only those with an active particle fulfill the requirement for active phage production ($Z = x e^{-x} e^{-r}$).

(c) Any bacterium that receives at least one active particle fulfills the requirement for active phage production, whether it also receives inactive particles or not. For each unit of that particle, $1 - e^{-r/n} = 0$; hence, $(1 - e^{-r/n})^k = 0$, and $[1 - (1 - e^{-r/n})^k]^n = 1$.

(d) For any given value of $r > 0$, Z increases with increasing x , that is, the probability of having a full complement of active units increases as the number of particles adsorbed per bacterium increases.

(e) For any given value of x , Z diminishes with increasing r , that is, the probability of having all active units diminishes as the dose of radiation increases.

For the purpose of comparison with data from different experiments, it is more convenient to eliminate from the computation those bacteria that receive either zero or one phage particle, since they are not expected to contribute to reactivation. This is done by using instead of Z the expression

$$z = \sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n. \quad (4)$$

z represents the fraction of bacteria in the total population that have two or more phage particles, which together contain a full complement of active units. Since the fraction m of bacteria with two or more phage particles ("multiple-infected bacteria") is

$$m = 1 - (x + 1)e^{-x} \quad (5)$$

the multiple-infected bacteria receiving a full complement of active units represent a fraction

$$y = z/m = \frac{\sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n}{1 - (x+1)e^{-x}}. \quad (6)$$

The expression y thus obtained is a function of x (average number of phage particles adsorbed per bacterium), of r (average number of lethal hits per particle), and of n (number of units per particle).

We shall call w the ratio between the number of bacteria that actually liberate active phage (plaque count) and the number of multiple-infected bacteria. For each mixture of bacteria and irradiated phage, we can determine experimentally r , x , and the plaque count, and obtain from these the values of m and w . We can then compare the experimental values of w with the calculated values of y for several different values of n .³

The function $y = F(r, x, n)$ was tabulated numerically for a range of values of r (between 3 and 50), of x (between 0.05 and 20), and of n (between 10 and 60). We used for k those ranges of values for which the contributions of the corresponding classes were relevant. The corresponding curves were drawn for $y = F(r)$ (x and n constant), and for $y = F(x)$ (r and n constant).

Before comparing the experimental results with the curves, it is useful to discuss briefly what we may expect from the comparison. If reactivation only occurs in bacteria with more than one inactive particle, w should never be greater than unity. If among the requirements for reactivation there are those stated in the assumptions of our theory, the ratio w/y should never be greater than unity. Finally, if the requirements stated in our assumptions are *necessary* and *sufficient* for reactivation, the ratio w/y should be unity, that is, active phage should be produced in all those bacteria that receive a full complement of the hypothetical units in non-lethal form. Should this obtain, it would then be possible to calculate the value of n for each phage from the experimental values of w .

It is important to keep in mind that the assumptions of our theory, up to this point, do not contain any implication as to the nature, properties, or mechanism of transfer of the postulated units. They only assert that each unit has genetic individuality and can be made lethal by radiation as a result of one photochemical reaction, which is of the "all or none" type and independent of other reactions of the same type in other units of either the same or other phage particles. Production of active phage is conditioned by the presence in one bacterium of one active copy of each unit, this copy not being replaceable by any number of inactive copies.

The theory does not imply that all phage particles receive the same number of lethal hits, but that the lethal hits are distributed at random among the units of all phage particles.

³ In preliminary reports (LURIA 1947, 1948) we used the symbol y both for the theoretical and experimental probabilities of reactivation. We also gave values of $1/y$ instead of y (or w). The present notation, while consistent with the previous one, makes the presentation more logical.

Comparison of theory with experiment

Quantitative experiments consisted of testing mixtures of bacteria and irradiated bacteriophage for the number of bacteria that liberate active phage. Only phages *T2*, *T4*, and *T6* were studied in detail.

In a typical experiment, such as the one described in detail in table 4, a standard culture of bacteria, grown to a titer of either 10^8 cells per ml or 10^9 cells per ml, was chilled by immersion in a water bath at $5-6^\circ\text{C}$. This treatment interrupts multiplication without changing the ability to resume immediate multiplication and to support normal growth of phage upon return to 37°C . In experiments with bacteria grown to a titer of 10^9 (latest part of logarithmic growth phase), immersion in ice-water is not necessary, since multiplication stops almost immediately upon interruption of aeration and transfer to room temperature.

Ten minutes after interrupting multiplication, a sample of the culture is diluted and assayed for viable count. If several mixtures of bacteria and phage are to be prepared, the culture may have to be used for one or two hours, in which case at least one other similar assay is made at the end of the experiment to make sure that no proliferation has occurred. At intervals, undiluted samples of the culture are placed into test tubes, and a constant volume of irradiated (or control) phage variously diluted is added. In this way, we know for each mixture the input of bacteria and of phage per ml. For irradiated samples, the input of active phage is determined from one or more assays done at very high dilution. When this is impossible—for high doses of radiation—the amount of active phage is determined by extrapolation from the first part of the inactivation curve.

Adsorption is interrupted by heavy dilution after a short time (generally five or ten minutes). The amount of adsorption is determined either by determination of free phage in the supernatant of a centrifuged sample from a mixture containing active phage, assuming similar adsorption in all other mixtures (see table 1), or by determining the bacterial survival in samples from various mixtures. With the *T-even* phages, 80 to 95 percent of the phage is adsorbed in ten minutes. The value for the multiplicity of infection, x , for each mixture is used in calculating the fraction m of bacteria with two or more phage particles: $m = 1 - (x+1)e^{-x}$.

Before lysis begins, a suitably diluted sample of the mixture is plated with an excess of sensitive bacteria for plaque count. The plaque count—corrected, when necessary, for the active phage by subtracting the value corresponding to the latter—is divided by m to obtain the experimental value w for that mixture (see table 4).

A large number of experiments, yielding a total of over 1000 values of w , for various doses of radiation and for different multiplicities, were done with the *T-even* phages. Phage *T5* was only partially investigated, because of difficulties in obtaining reproducible results in view of the low and irregular adsorption rate for this phage.

The data for phages *T-even* include those for several of their r mutants,

TABLE 4

Reactivation of phage T2r in a series of mixtures of irradiated phage with bacteria.
Sample experiment, being a part of Experiment No. 155

Culture of *E. coli* B chilled at 6°C = (B). Bacterial titer by plate count: 1.1×10^8 cells per ml.

Phage T2r (stock No. 18c diluted 1:4) irradiated for 20, 30, 40, and 50 seconds.

Control phage, diluted 1:10⁷; 0.1 ml gives 220 plaques. Titer = 2.2×10^{10}

Phage irradiated 20 sec., dil. 1:10⁴; 0.1 ml gives 483 plaques. Titer = 4.8×10^7 . Survival ($e^{-\tau}$) = 2.2×10^{-3} . $r = 6.1$.

Phage irradiated 30 sec. dil. $1:4 \times 10^3$; 0.1 ml gives 36 plaques. Titer = 1.45×10^6 . Survival ($e^{-\tau}$) = 6.6×10^{-4} . $r = 9.6$.

MIX- TURE NO.	CONTENTS	PLAQUES PER ML OF MIXTURE (SAMPLES PLATED AFTER 10 MINUTES AT 37°C, WITH SUITABLE DILUTION)	PHAGE INPUT	ADSORBED* PHAGE	MULTI- PLICITY OF INFECTION x	MULTIPLE- INFECTED BACTERIA m	PLAQUE COUNT**	PLAQUE COUNT m	$=w$
1	0.1 ml (Phage irradi. 20 sec. dil. 1:2.5)+0.9 ml (B)	9×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	9×10^7	0.9	
2	0.1 ml (Phage irradi. 20 sec. dil. 1:10)+0.9 ml (B)	2×10^7	2.2×10^8	2.05×10^8	2.05	5.5×10^7	2×10^7	0.36	
3	0.1 ml (Phage irradi. 30 sec. dil. 1:2.5)+0.9 ml (B)	7.5×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	7.5×10^7	0.75	
4	0.1 ml (Phage irradi. 30 sec. dil. 1:10)+0.9 ml (B)	1×10^7	2.2×10^8	2.05×10^8	2.05	5.5×10^7	1×10^7	0.18	
5	0.1 ml (Phage irradi. 30 sec. dil. 1:100)+0.9 ml (B)	2.2×10^6	2.2×10^7	2.05×10^7	0.20	1.6×10^6	2.2×10^6	0.14	
6	0.1 ml (Phage irradi. 40 sec. pure)+0.9 ml (B)	8.8×10^7	2.2×10^8	2.05×10^8	20.5	1×10^8	8.8×10^7	0.88	
7	0.1 ml (Phage irradi. 40 sec. dil. 1:2.5)+0.9 ml (B)	4.3×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	4.3×10^7	0.43	
8	0.1 ml (Phage irradi. 40 sec. dil. 1:10)+0.9 ml (B)	4.7×10^6	2.2×10^8	2.05×10^8	2.05	5.5×10^7	4.7×10^6	0.085	
9	0.1 ml (Phage irradi. 40 sec. dil. 1:100)+0.9 ml (B)	4×10^4	2.2×10^7	2.05×10^7	0.20	1.6×10^6	4×10^4	0.025	
10	0.1 ml (Phage irradi. 50 sec. pure)+0.9 ml (B)	5.4×10^7	2.2×10^8	2.05×10^8	20.5	1×10^8	5.4×10^7	0.54	
11	0.1 ml (Phage irradi. 50 sec. dil. 1:2.5)+0.9 ml (B)	2.3×10^7	8.8×10^8	8.2×10^8	8.2	1×10^8	2.3×10^7	0.23	
12	0.1 ml (Phage irradi. 50 sec. dil. 1:100)+0.9 ml (B)	5.4×10^3	2.2×10^7	2.05×10^7	0.20	1.6×10^6	5.4×10^3	0.0034	
13	0.1 ml (Phage Control dil. 1:100)+0.9 ml (B) Free in supernatant	2.1×10^7 1.5×10^6							

* Determination of adsorption. Mixture No. 13:

Adsorbed phage = $2.2 \times 10^7 - 1.5 \times 10^6 = 2.05 \times 10^7$

Fraction adsorbed = 0.93

Bacteria per tube = 1×10^8

Multiplicity of infection = 0.205

Infected bacteria (calculated) = 1.85×10^7

Infected bacteria + free phage = 2.0×10^7

Plaque count (experimental) = 2.1×10^7

** No correction is made for active phage in the input, since this correction would only affect the second (or higher) decimal figure.

TABLE 5

Probability of reactivation for various phages as a function of dose and of multiplicity of infection

Values of the ratio w between bacteria that yield active phage and multiple-infected bacteria.

EXPERI- MENT NO.	PHAGE	MULTI- PLICITY x	AVERAGE NUMBER OF HITS PER PARTICLE							
			r	2.6	4.8	6.0	9.5	10.0	12.5	15.8
155	$T2r$	0.2					0.14		0.025	0.0034
		0.4							0.028	0.0055
		1							0.055	0.01
		2				0.36	0.18		0.085	
		4							0.18	0.08
		8				1.0	0.83		0.48	0.26
		20							0.98	0.6
		0.1			0.56	0.42		0.1		
		0.2		(1.0)	(0.83)	0.40		0.1		
		1		(0.9)	(0.59)	0.44		0.17		
156	$T2r$	2				0.5		0.23		
		4				0.67		0.43		
		8				1.05		0.74		
		20				1.0		1.0		
			r	3.6	6.1	7.9	9.6	11.7	12.8	15.5
		0.2					0.065			
162	$T6r$	0.4			0.15		0.075		0.03	
		0.8			0.21		0.1		0.035	
		2			0.27		0.18		0.05	
		4			0.59		0.26		0.18	
		8			0.83		0.67		0.37	
		0.35	(1)		0.27		0.07			0.016
70	$T6$	0.7	(1)		0.37		0.08			0.016
		1.4			0.37		0.12			0.04
		2.8			0.37		0.14			0.06
		5.6			0.48		0.21			0.08
			r	5			10			
160	$T4$	0.35			0.4		0.027			
		0.7			0.29		0.042			
		1.4			0.28		0.06			
		2.8			0.42		0.13			
		7			0.8		0.38			
		14			1.0		0.77			

The values in parentheses are from separate experiments.

which were found to have the same probability of reactivation as the respective wild types. The results cover ranges of values of r from 2.5 to over 30, and of x from 0.02 to 20.

The individual values of w , and those of the variables, r and x , are obtained from the following actual measurements: 1) titer of phage; 2) total number of bacteria; 3) survival of phage; 4) survival of bacteria and/or assay of free phage; 5) plaque count from the mixture. Each of these measurements involves an error of estimation due to dilution and sampling errors. Several of these determinations, however, are the same within each experiment. The results from individual experiments are, therefore, more consistent than those from different experiments, as shown in table 5.

The only graphic representation that could show all values of w for each phage and allow of comparison with the calculated values of y would be a tri-dimensional plot of w as a function of r and of x . As second best choice, we plotted the values of w as a function of r for several values of x taken as constant, and as a function of x for several values of r taken as constant. Individual values of w fluctuate rather widely, but the data as a whole make it possible to draw curves, which represent averages and which can be considered as the curves for w as a function of r and of x . A number of such plots using all the experimental points for the corresponding values of the variables, are presented in figures 2, 3, and 4 (multiplicity of infection as variable) and figures 5, 6, and 7 (dose of radiation as variable).

The trend of these plots is similar to that of the theoretical curves for y , and it is possible to find for each phage a constant value of n (number of units) such that the corresponding values of y become very similar to those of w for low values of x and for any value of r . That is, it is possible for each phage to determine a constant number of units for which the experimental probability of reactivation equals the theoretical one for any dose of radiation provided the multiplicity of infection is low. The corresponding theoretical curves for y have been drawn in the plots of the values of w . For phage *T2*, the best fit is for $n=25$; for *T4*, $n=15$; for *T6*, $n=30$ (see especially figures 5, 6, and 7).

The main feature emerging from the curves in figures 2, 3, and 4 is that the values of w tend to unity for increasing multiplicity of infection. In several cases, the number of cells that liberate phage actually reaches the number of multiple-infected cells, but in no case does it go beyond it, proving that reactivation does not occur in single-infected cells.

The curves in figures 5, 6, 7, for w as a function of r , are of the multiple-hit type, indicating that suppression of phage production depends on damage in a number of elements. The values of w tend to unity for low doses, again showing that reactivation potentially can take place in every multiple-infected cell.

Comparison in figures 2-4 with the curves for y , chosen to fit the experimental curves for low values of x , shows that the general similarity is limited by a systematic deviation. As the multiplicity increases, both w and y tend asymptotically to unity, but w increases more slowly. This means that, as the number of phage particles per bacterium increases, the probability of reactivation

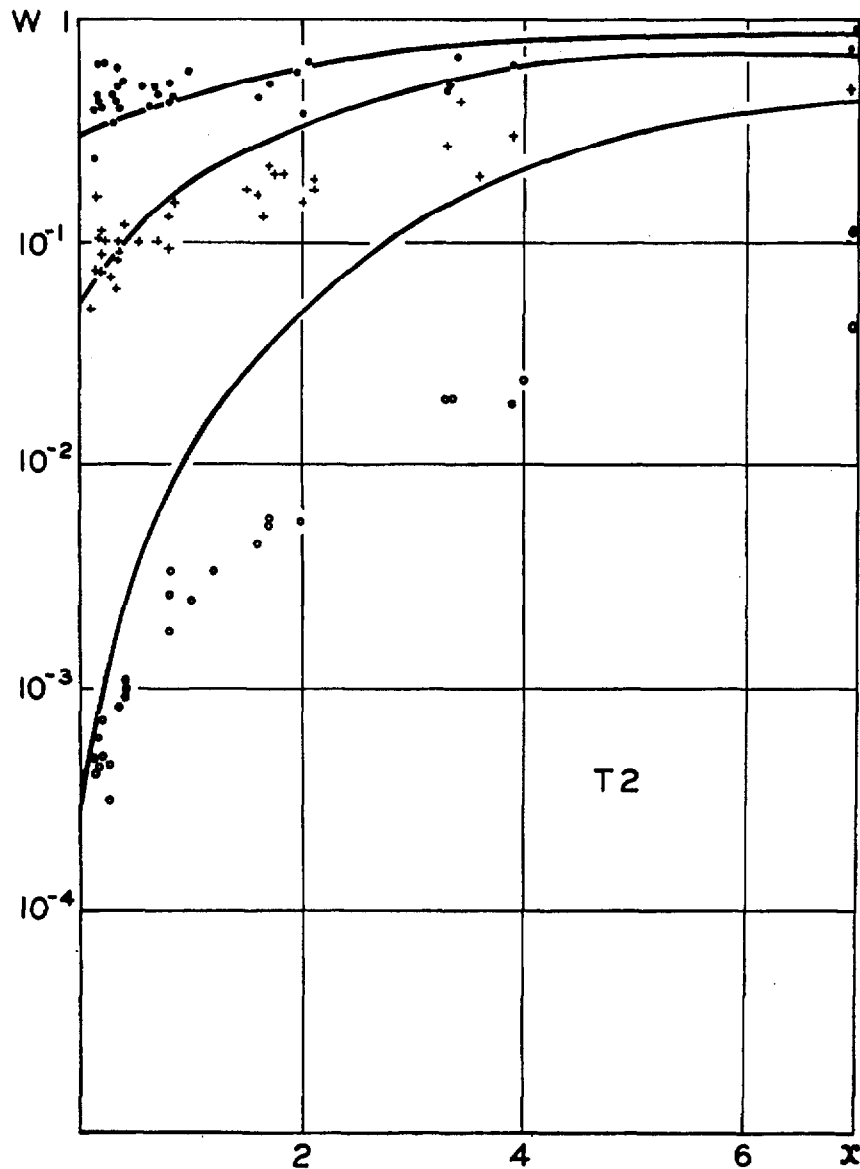


FIGURE 2.—The probability w of reactivation for irradiated phages $T2$ and $T2r$ as a function of the multiplicity of infection x , for several doses of radiation. Abscissae: values of x . Ordinates: values of w .

- values of w for $r=6$ hits per particle.
- + values of w for $r=10$ hits per particle.
- values of w for $r=20$ hits per particle.

Solid lines: theoretical curves for y as a function of x for $n=25$, and for the values of r given above.

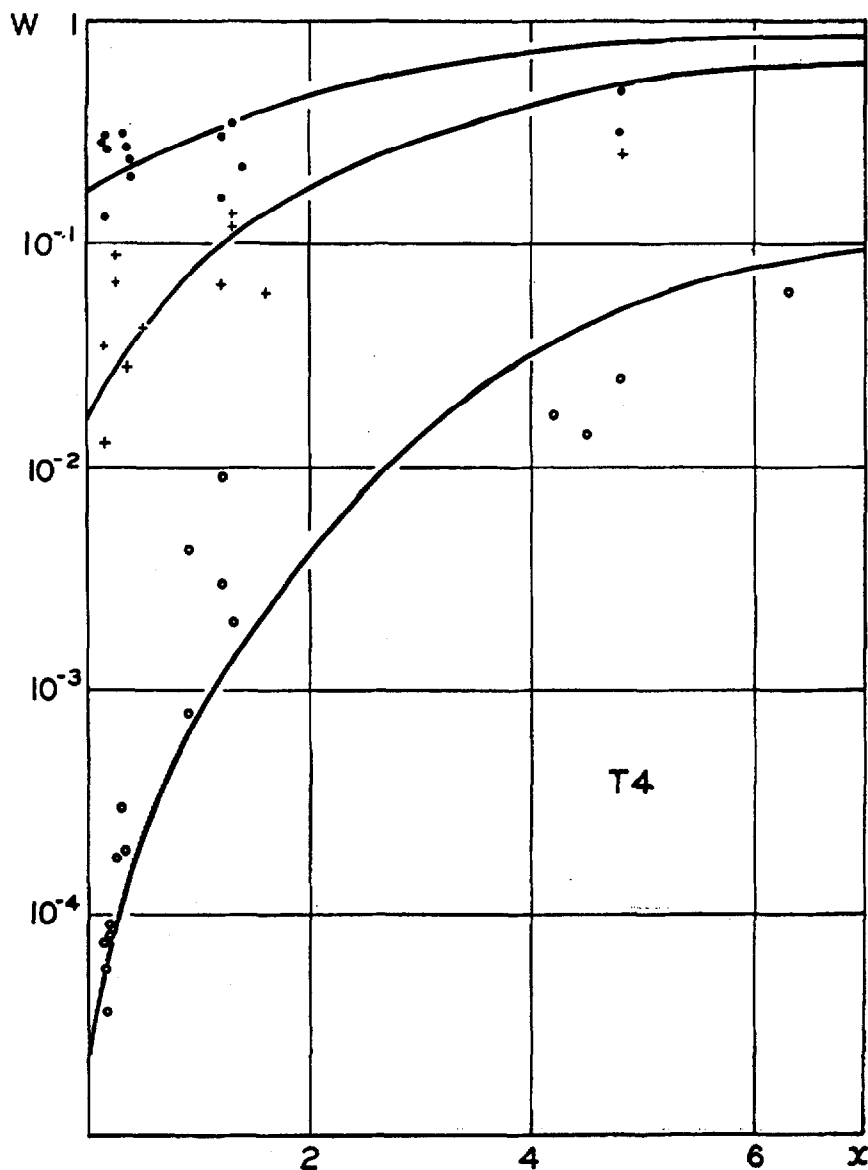


FIGURE 3.—Same as figure 2, for phages *T4* and *T4r*. The theoretical curves for y correspond to $n=15$.

vation, while steadily increasing, does not keep pace with the theoretical function y . The cooperation within groups consisting of more than two particles is not as successful in bringing about reactivation as required by the simple theory, whereas pairs of particles apparently collaborate with an efficiency of one hundred percent.

The deviation for higher multiplicities is reflected in the curves of figures 5-7. The curves for w as a function of the dose are very close to the theoretical

curves for low multiplicities, up to $x=0.5$; for higher multiplicities they fall below the corresponding curves for y calculated for the same number of units. We can actually find, for each value of x , a curve for y —for the same n but corresponding to a lower x —which fits the experimental curve. The corresponding theoretical curves are drawn in figures 5–7. This indicates that groups of more than two particles collaborate in reactivation as if they consisted of a lower but definite number of particles.

It is interesting to notice that the systematic deviation from theory for

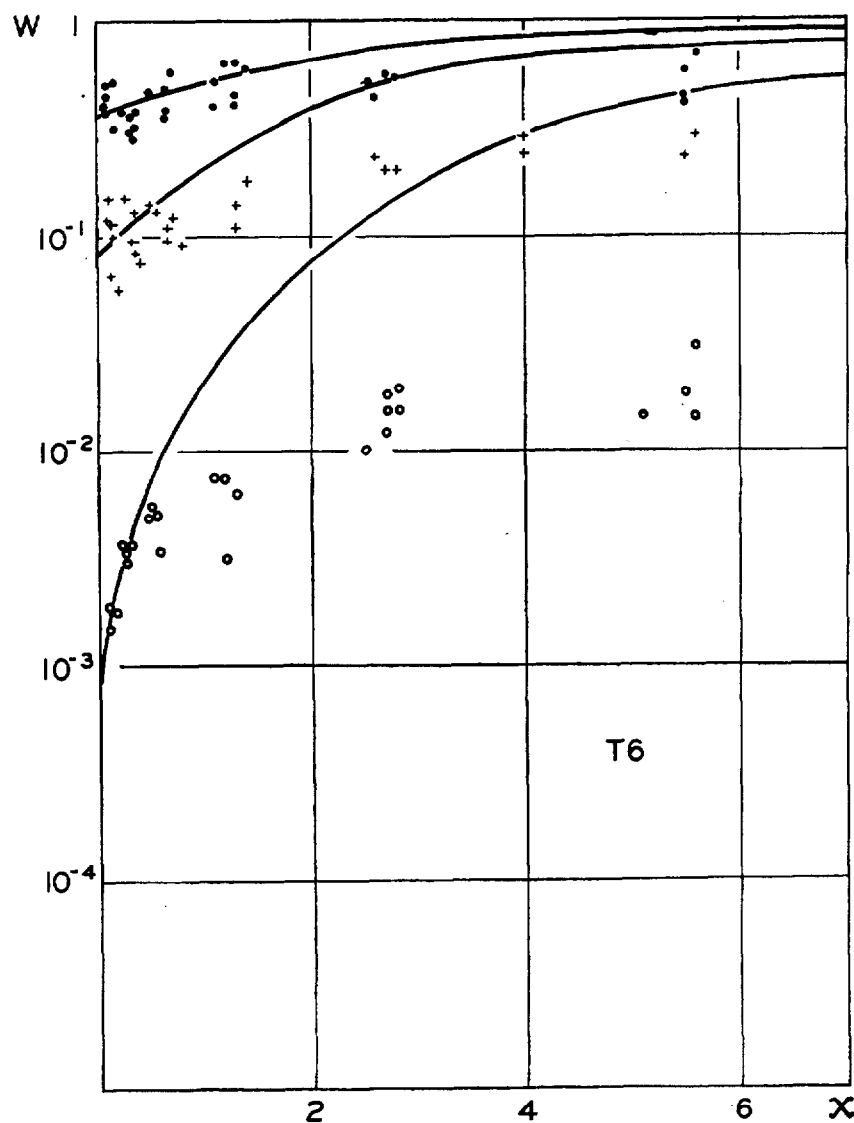


FIGURE 4.—Same as figure 2, for phages *T6* and *T6r*. The theoretical curves for y correspond to $n=30$.

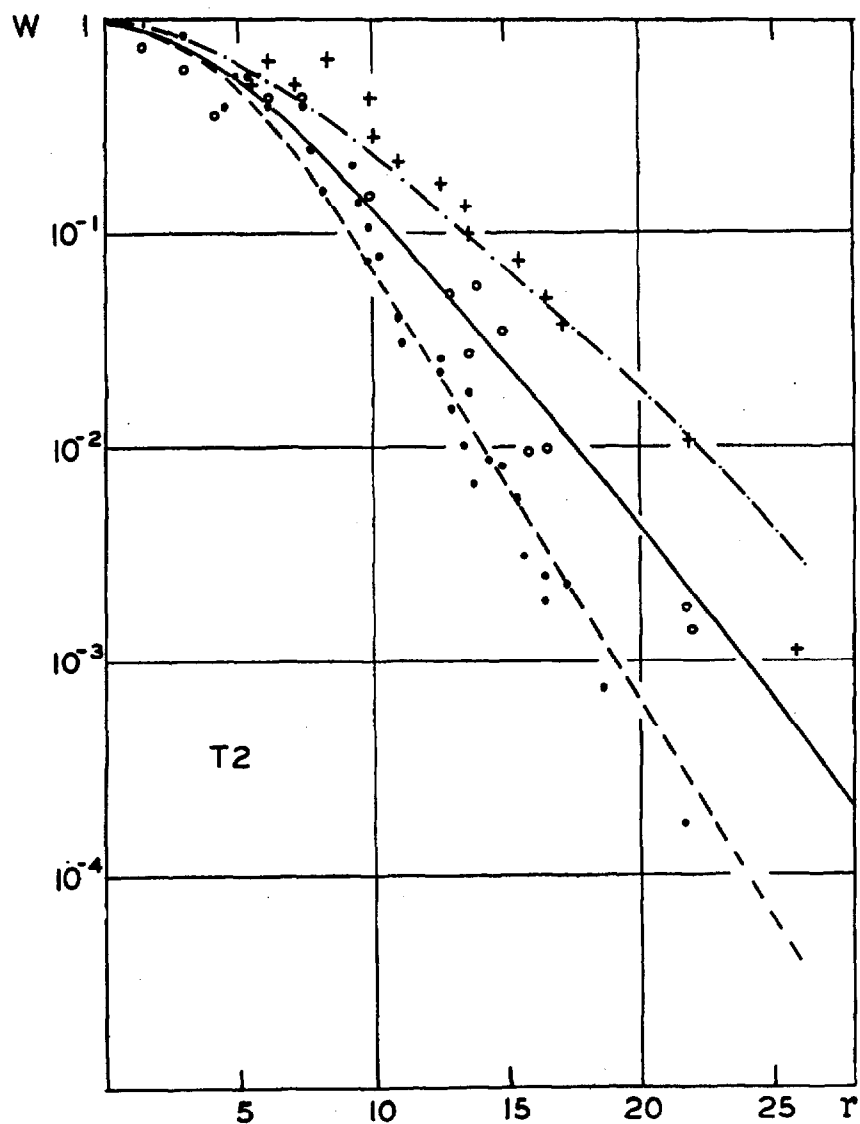


FIGURE 5.—The probability w of reactivation for phages $T2$ and $T2r$ as a function of the dose of irradiation r (in hits per particle) for several multiplicities. Abscissae: values of r . Ordinates: values of w .

- values of w for $x=0.1-0.2$.
- values of w for $x=0.8-1.5$.
- + values of w for $x=2.5-4.0$.

Broken line: theoretical curve for y as a function of r for $n=25$, $x=0.15$. Solid line: theoretical curve for y as a function of r for $n=25$, $x=0.6$. Broken and dotted line: theoretical curve for y as a function of r for $n=25$, $x=1.3$.

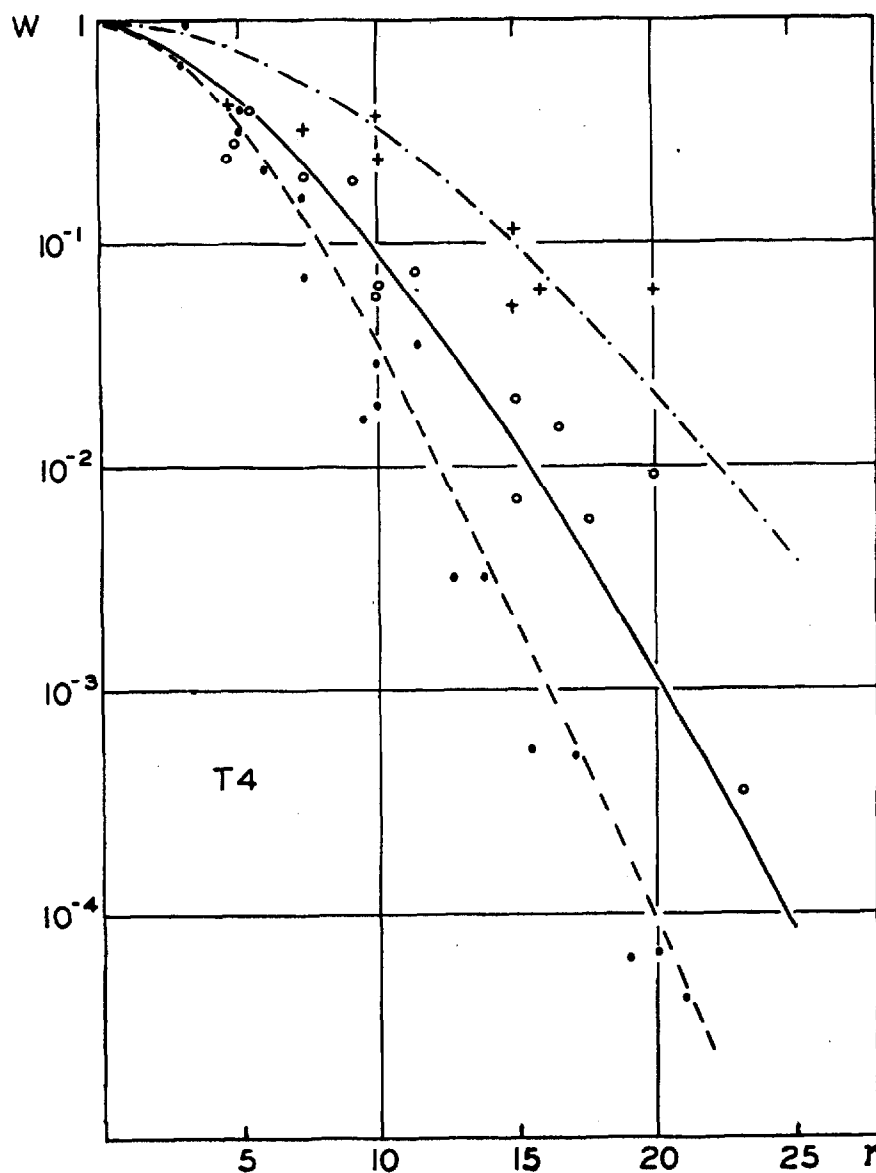


FIGURE 6.—Same as figure 5, for phages *T4* and *T4r*.

- values of w for $x=0.1-0.2$.
- values of w for $x=0.8-1.5$.
- + values of w for $x=4-7$.

Broken line: curve of y for $n=15$, $x=0.15$. Solid line: curve of y for $n=15$, $x=1.1$. Broken and dotted line: curve of y for $n=15$, $x=4.0$.

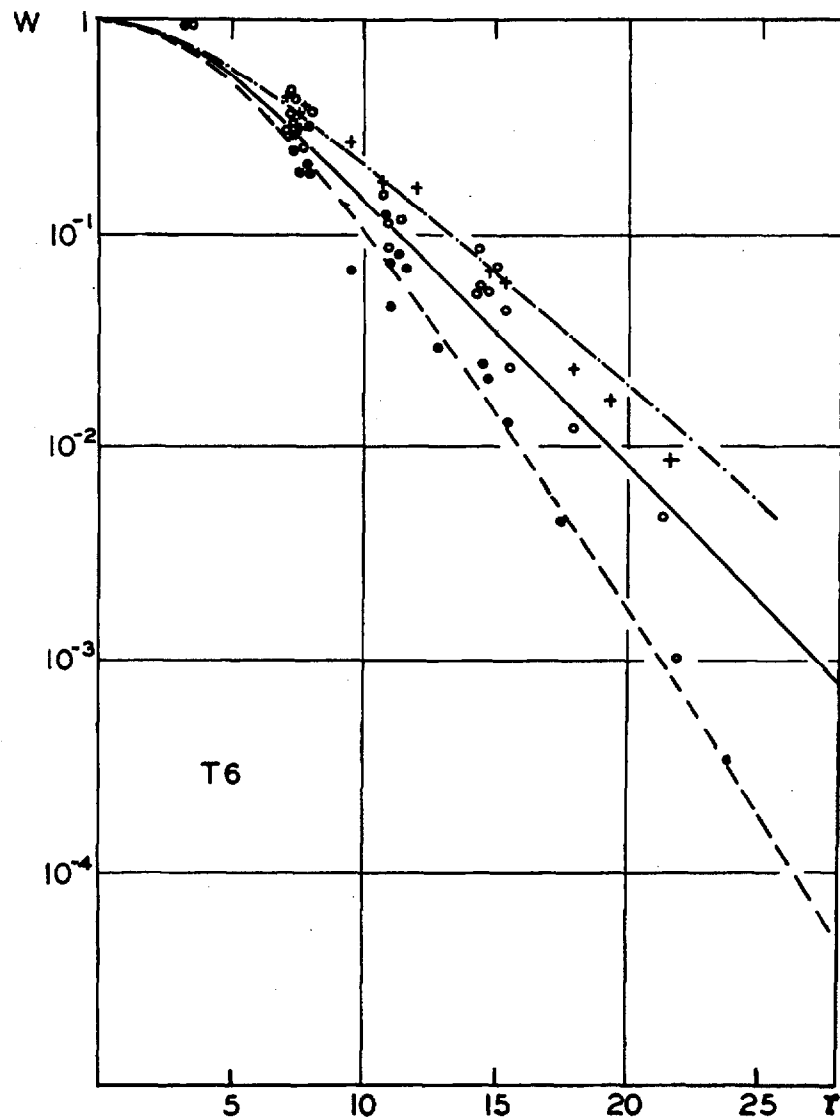


FIGURE 7.—Same as figure 5, for phages *T6* and *T6r*.

- values of w for $x=0.1-0.2$.
- values of w for $x=0.8-1.5$.
- + values of w for $x=2.5-4.0$.

Broken line: curve of y for $n=30$, $x=0.15$. Solid line: curve of y for $n=30$, $x=0.5$. Broken and dotted line: curve of y for $n=30$, $x=0.9$.

increasing multiplicities is less evident for phages *T2* than for *T6*, and still less for *T4*, diminishing in the same order as the calculated number of units.

To summarize, the results show that the probability of production of active phage from inactive particles depends on the dose of radiation and on the multiplicity of infection in a way similar to the one predicted by the simple theory, which assumes lethal mutations in discrete transferable units of equal radiation sensitivity and a hundred percent efficient recombination of active units to reconstitute active particles. All deviations can be accounted for by a limitation in the efficiency of recombination when the active units derive from more than two inactive particles.

Several explanations may be offered for this limitation; some of them have been tested experimentally. Lysis from without—failure to liberate phage due to excessive multiplicity of infection (DELBRÜCK 1940)—was found not to take place for multiplicities of the order of those for which the deviations from theory occur. Limitations in the number of particles of a given phage that can participate in phage growth were looked for and found (see DULBECCO 1949a), but their magnitude cannot account for the differences between w and y .

The process of reactivation must involve complex mechanisms of transfer of genetic material among phage particles. Whatever these mechanisms, it is reasonable to expect that they will work less efficiently as the number of phage particles increases. Limitations may conceivably be caused by steric reasons—shape of the particles, position in the bacterium—or by physiological reasons—limited number of units of some catalyst, competition for substrates.

Estimation of the number of units

We have compared our results with the theoretical curves for y calculated for different values of n , the unknown number of transferable units per particle. The curves that best fit the results for phages *T2* and *T2r* are those for $n=25$; for *T6* and *T6r*, $n=30$; for *T4* and *T4r*, $n=15$. For phage *T5*, no accurate estimate of n was obtained, but n appears to be lower than for *T4*.

If the interpretation of the results based on the simple theory is justified, we must consider the values thus obtained for n as minimum estimates of the number of radiation-sensitive, transferable units per phage particle. The estimates are minima because of the assumption of equal sensitivity of all units. Should there be units more sensitive than others, they would be hit more often, and in order to obtain the correct probability of reactivation we should assume more units of the less sensitive type. For example, if one unit were twice as sensitive as the average of the others, one locus of the average sensitivity should be added to our estimate in order to distribute the probability of inactivation over all units in such a way that the reactivation probability remains the same.

In our preliminary report (LURIA 1947) we calculated n in a different manner, by assuming that for low multiplicities and low doses, where the probability of reactivation appeared to be approximately constant as a function of x , we could consider all multiple-infected bacteria as double-infected. This corre-

sponded to putting $k = 2$ in formula (6) and to comparing the experimental values of w with the values of y for $x = 0$. Upon closer analysis, this method proved incorrect, because the contribution of higher multiple infection cannot be neglected, even for low multiplicities. Analysis of more data showed that the probability of reactivation is in fact not constant for low values of x , but appears to be so for low doses, because the differences are small and of the order of the experimental errors. The use of the wrong approximation made our previous estimates of n too high.

Yield of active phage from bacteria in which reactivation occurs

The yield of active phage following reactivation was studied systematically for phages *T2r* and *T4*. After infection, bacteria were diluted and allowed to lyse in liquid, as in a typical "one-step growth" experiment. The latent period before lysis is somewhat longer than for active phage (about 26 minutes instead of 21 for *T2*, 30 minutes instead of 25 for *T4*), and the rise in phage titer upon liberation somewhat slower. All yields were calculated from plaque counts after the titer had reached a steady level. The results, shown in table 6, indicate that the yields are generally somewhat lower than those from bacteria infected with active phage particles. No clear relation of yield to dose of radiation or to probability of reactivation was detected. For *T2r* irradiated with high doses, there is a certain tendency toward higher yields for higher multiplicities.

Mixed infection with active and inactive phage

Transfer of genetic material involved in reactivation must occur between active and inactive phage particles, since, as we saw before, an active particle of a *T-even* phage can reactivate an inactive particle of another *T-even* phage. If transfer occurred by reciprocal exchanges of genetic material, we should expect that upon mixed infection with active and inactive particles of the same phage some of the active particles would receive inactive units and, therefore, be inactivated. This possibility was tested for phages *T2* and *T4* by experiments of the following type.

Bacteria are added to mixtures containing various proportions of active phage and of phage of the same strain irradiated with different doses. For each mixture, the average numbers of active and of inactive particles adsorbed per bacterium are calculated and, hence, the number of bacteria receiving both active and inactive phage. A plaque count before lysis gives the number of bacteria that liberate phage, while a plaque count after lysis gives the yield of phage per bacterium. In this manner, we can determine whether inactive phage suppresses production of active phage from bacteria that also adsorb an active particle, or possibly affects the yield.

The results of these tests can be listed as follows:

- (a) a bacterium receiving an active particle plus one inactive particle of the same phage—no matter how many hits the latter has received—never fails to liberate active phage;
- (b) part of the bacteria that adsorb one active particle plus several inactive

ones (the latter carrying enough lethal hits, so that reactivation does not occur among them) fail to liberate active phage. This suppression of active phage production is evident for multiplicities 5 or higher of inactive phage *T4*, and for multiplicities 8 or higher of inactive phage *T2*. The suppression is independent of the time allowed for adsorption of the phages. It may in part be

TABLE 6
The yield of active phage from bacteria in which reactivation takes place

PHAGE	MULTIPLICITY OF INFECTION	DOSE				
		HITS	0	6	12	18
<i>T2r</i>	0.2-0.25		114	51	122	
	0.5-0.6		50	42	22	
	2.9		89		100	29
	7.7		59			60
	9		100	70	94	
	10-11		73	120	59	130
		HITS	0	7.5	15	18
<i>T4</i>	0.65		260	120	87	200
	1.1		200	87	162	
	1.6		275		140	140
	2.7		212		197	
	7-8				135	200
	14-15				250	85

accounted for by the limitation phenomenon described by the junior author (DULBECCO 1949a);

(c) for those bacteria that liberate active phage, the yield per bacterium is not affected by the presence of inactive particles, but remains the same as in controls without the inactive phage;

(d) when bacteria are infected with several heavily irradiated particles that do not give reactivation, and a few minutes later with one active particle, suppression of phage production occurs in a proportion of bacteria that increases with the interval between infections. Suppression is evident with an interval of 2.5 to 4 minutes and practically complete after 10 minutes. These time intervals between infections are, of course, averages, since infection may occur earlier or later for individual bacteria in the same mixture. The yield from those bacteria that liberate phage still remains normal.

The suppression of active phage reproduction by inactive phage in excess indicates the existence of some type of "mutual exclusion" between particles of the same phage. Such exclusion is also indicated by the experiments of DULBECCO (1949a).

More detailed analysis of the interaction between active and inactive phage, using genetic markers, will be reported in future papers. Our results

discussed above are in agreement with earlier observations (LURIA and DELBRÜCK 1942) on interference by a large excess of irradiated phage *T2* with the growth of active phage *T2* when the inactive phage was mixed with bacteria one minute and a half before the active one.

Cross-reactivation between phage particles differing by one character

Only one group of experiments will be discussed here, because of its bearing on the analysis of the data presented in this article. Bacteria were infected with

TABLE 7
*Cross-reactivation between one inactive particle of phage T2 and
one inactive particle of phage T2r*

Compare column (3) with column (1)

EXPERI- MENT NO.	DOSE OF RADIATION, HITS	MULTIPLIC- ITY OF INFECTION FOR <i>T2</i>	MULTIPLIC- ITY OF INFECTION FOR <i>T2r</i>	(1) FRACTION OF BACTERIA RE- CEIVING <i>one</i> INACTIVE PAR- TICLE <i>T2</i> AND <i>one</i> INACTIVE PARTICLE <i>T2r</i> AMONG THE BACTERIA THAT LIBERATE AC- TIVE PHAGE, CALCULATED	(2)* OTHER BAC- TERIA THAT COULD GIVE MOTTLED PLAQUES (AS FRACTION OF THE BACTERIA THAT LIBERATE ACTIVE PHAGE), CALCULATED	(3) FRACTION OF MOTTLED PLAQUES, FOUND
1A	2.9	0.055	0.055	0.19	0.045	0.14
2A	4.6	0.055	0.049	0.335	0.053	0.16
3A	6.2	0.055	0.055	0.42	0.074	0.155
4A	3.4	0.053	0.053	0.24	0.046	0.14
5A	5.0	0.053	0.053	0.355	0.068	0.20
7A	3.9	0.05	0.05	0.28	0.046	0.14
8A	6.5	0.05	0.05	0.19	0.087	0.21

* The values in this column include all bacteria with two or more particles of one type and one or more of the other type, plus all bacteria with an active particle of one type and an inactive particle of the other. The values are upper limits, since only a fraction of these bacteria will actually give mottled plaques.

phages *T2* and *T2r*, both irradiated ($r=5$ or 6). Low multiplicities were used, so that a large proportion of the infected bacteria only received one inactive particle, and, of those that received two, a great proportion received one particle of each type. The infected bacteria were plated before lysis, and the plaques examined for the proportion of "mottled plaques," that is, of plaques containing both *T2* and *T2r* active phages. Such plaques can only arise from bacteria infected with both phages. It is seen from the data shown in table 7 that more than half the bacteria infected with one inactive particle of each of the two phages actually liberate a mixture of active particles of both types.

This proves that recombination cannot result from reciprocal exchanges of

units between the infecting particles bringing together into one particle all the active units before multiplication begins. Evidently, this particle should be either *T2* or *T2r*, and could not give rise to active particles of both types. This conclusion will be analyzed further in the discussion. Quantitative analysis of the number and contents of mixed yields from mixed infection with *T2* and *T2r* shall be the subject of future publications.

Phages inactivated by X-rays or nitrogen mustard

In a preliminary article (LURIA 1947) it was stated that no reactivation had been detected for *T-even* phages inactivated by hard X-rays, using the same technique employed for ultraviolet. The same was found in our laboratory by MISS M. E. WILLIS for phages *T2* and *T6* inactivated by a nitrogen mustard (methyl bis (β -chloroethyl) amine hydrochloride).

Experiments by MR. J. WATSON, still in progress in our laboratory, have recently shown, however, that phage *T2* inactivated by hard X-rays can take part in reactivation, but this reactivation occurs with such a low probability that special techniques are required for its detection. In part, the low probability of reactivation of X-ray inactivated phage is due to reduced rate of adsorption. This work will be reported by Mr. WATSON in a future publication.

It seems possible that phages *T1* and *T7*, for which no reactivation was detected after ultraviolet inactivation, may also be found by similar techniques to give some reactivation. It is clear that the probability of reactivation will be low if the number of transferable units is small, or if each lethal mutation involves several units. Its detection will be difficult whenever the number of bacteria in which reactivation occurs is small in comparison with the number of bacteria that receive residual active phage.⁴

DISCUSSION

The experiments described above have given results consistent with the hypothesis that inactivation of several and possibly all bacteriophages by ultraviolet light is to be attributed to lethal mutations in discrete units of genetic material. A genetic basis for inactivation of viruses and bacteria by radiation has often been postulated either on statistical grounds or by analogy (see RAHN 1929; LEA 1947). Our results bring new support to this view, and suggest that most, if not all, the inactivating effect of ultraviolet light (2537 Å) on certain phages is due to the production of localized lethal mutations.

The hypothesis of inactivation by lethal mutations and reactivation by transfer of genetic material following multiple infection, as developed in this paper, has been useful in suggesting a quantitative analysis of the reactivation phenomena and has led to fairly accurate predictions of the experimental results. The following discussion assumes the correctness of this working hypothesis.

⁴ While this paper was in press, the senior author found that some reactivation by multiple infection takes place with phage *T1* inactivated by ultraviolet light. For equal multiplicity of infection and equal number of hits, the frequency of reactivation is much lower with *T1* than with any of the *T-even* phages and with *T5*. Assuming that the type of analysis presented in this paper applies to the results with *T1*, a value of *n* smaller than 5 would be obtained.

According to our analysis, active phage is reconstituted from inactive by re-incorporation of active units derived, directly or indirectly, from the inactive particles in a kind of hybridization. As in hybridization, the possibility of recombinations between particles of different wild-type phages suggests the existence of common genetic determinants and the absence of complete incompatibility. It is possible that various interference phenomena among different phages may result from such an incompatibility.

Reassembly of material from inactive particles into active ones is a remarkably efficient process. Genetic material from several inactive particles may be brought together, although the relative efficiency of cooperation diminishes as the number of particles involved in this pluriparental reproduction increases.

We have given estimates for the minimum number of transferable units per particle for several phages. It is interesting to notice that phage *T4*, more resistant than the related phages *T2* and *T6* to ultraviolet light (figure 1), appears to have fewer units. This may indicate absence of a portion of genetic material present in the other *T-even* phages.

LEA and SALAMAN (1946), analyzing the dependence of the rate of inactivation of phages by X-rays as a function of the density of ionization, and assuming that the radiosensitive material consisted of spherical units, arrived at the conclusion that a large phage contained 14 such units, whereas a small phage contained one only. Although the hypotheses involved were probably oversimplifications, the conclusion receives qualitative support from our results.

We must consider next the possible mechanisms of genetic transfer. We may divide the mechanisms into two groups, those in which the reproducing element is supposed to be at all times the phage particle as a whole, and those in which the reproducing elements are assumed to be component parts of the particle.

The simplest hypothesis of the first group would be that reactivation results from pairing (or grouping) of the initial infecting particles, followed by reciprocal exchanges such as occur in chromosomal crossing-over; if these exchanges lead to formation of an active particle, the latter proceeds to multiply. This simple hypothesis can easily be disproved. The high efficiency of reactivation would require very large numbers of successive reciprocal exchanges to bring together all active genetic material before multiplication takes place. Mixed infection with one active and one inactive particle should also lead to the occasional loss of active phage, since we know that genetic recombinations occur between active and inactive particles. Finally, the fact that infection with one particle each of inactive *T2* and inactive *T2r* yields a mixture of active *T2* and active *T2r* disproves this hypothesis, since any number of reciprocal exchanges between the original particles *before multiplication* could never lead to formation of active particles of both types.

Another interpretation based on reciprocal exchanges would be that inactive particles reproduce, and that exchanges occur at various stages of the reproduction among the original particles or their inactive offspring. These exchanges should be numerous enough to make the probability of incorporation of all active units into an active particle close to unity, and an ac-

tive particle, once formed, should be favored in multiplication. Without completely disproving it, our results make this explanation very unlikely. The fact that the probability of reactivation increases greatly by increasing (for example, from 8 to 20) the multiplicity of infection with heavily irradiated particles would require a very high number of exchanges. To obtain high yields of active phage in these cases we should assume, moreover, that the exchanges take place early, and that the active particles, once formed, multiply with little or no interference from the inactive particles in large excess. This seems contradicted by the fact already mentioned that active particles can actually undergo interchanges with inactive ones. Altogether, there is strong evidence that inactive units have less chance than active units of entering the final particles, even when the active units derive from inactive particles.

In search for a mechanism that could selectively bring together the active units, the senior author (LURIA 1947) suggested the hypothesis of independent reproduction of individual units to form a "gene pool," from which the new active particles could be derived. Inactive units were considered to be those that cannot reproduce and that have, therefore, little chance of incorporation into the final particles. The tendency to reduction in yield may be due to occasional incorporation of some of the original inactive units. No hypothesis is made as to how the units reproduce or reassemble. The last step is the most difficult to visualize, and we incline to the belief that the original particles may play a role in it, possibly by supplying a framework for reassembly. This is suggested by the limited efficiency of collaboration among large groups of particles, which indicates a certain tendency of the units to remain together with their original companions.

One may ask whether, inside bacteria in which reactivation does not take place, the active units present in the infecting particles reproduce or not. COHEN (1948) states that no desoxyribose nucleic acid is synthesized in bacteria infected with particles of *T2* exposed to doses of ultraviolet light much higher than those employed in our study. Preliminary cytological evidence, collected with the collaboration of Dr. C. F. ROBINOW in our laboratory, indicates that in infected bacteria, in which reactivation does not take place, there is no accumulation of stainable material supposedly representing desoxyribose nucleotides. If this evidence is confirmed and found to apply to the conditions of our experiments, it might then suggest, either that there is no reproduction of active units when they are not all present (which might altogether invalidate the hypothesis of independent reproduction), or that at least part of the reproduction of the active units may take place without increase in desoxyribose nucleotides.

The hypothesis of a "gene pool," although by no means the only possible one,⁵ fits all results of reactivation. Its validity may soon be amenable to

⁵ Another possibility, suggested by Dr. A. H. STURTEVANT, would be a process of zipperwise replication of the various units of a phage particle. When in this process an inactive unit was reached, replication could only continue if the partial replica came in contact with another phage particle in which that unit was active. The process would then continue by addition of replicas of the active units of the second phage particle. If repeated several times, such a mechanism would provide for selective recombination of all active units.

critical test. In postulating a phase in phage growth in which particles, as we know them in the extracellular phase, are not present, the hypothesis accounts for the repeated failures to obtain active phage by premature artificial disruption of infected bacteria. It agrees with the observation made by FOSTER (1948) in our laboratory that in the presence of proflavine the reactions leading to production of active phage proceed normally for a part of the latent period, but, upon lysis, no active particle is liberated. According to FOSTER (1948) active phage particles are present in the infected bacterium after the proflavine sensitive stage is passed—beginning 12 to 14 minutes after infection for phages *T2* or *T6*. A similar conclusion was reached by A. H. DOERMANN on the basis of experiments on the effect of other inhibitors on the growth of *T3* and *T4r* (DOERMANN 1948). That viruses multiplying inside the host cell may not have the same organization as in the extracellular form has been suggested before for certain animal viruses (see BLAND and ROBINOW 1939).

Our theory does not assume any degree of linkage among units, although linkage may be compatible with the theory. Each group of strongly linked units would behave as one unit, possibly as a particularly sensitive one. Weakly linked units would probably reduce the probability of reactivation for high doses, when lethal units present in one group would hinder the utilization of the linked active units for reactivation.

In their work on the *r* and *h* mutants of phage *T2 H*, HERSHEY and ROTMAN (1948, 1949) found evidence for a series of determinants exhibiting various degrees of linkage, from those apparently unlinked (high frequency of independent transfer, no correlation between the frequencies of complementary recombinant types in the yield) to others rather strongly linked. For the latter ones, the authors considered that their results suggested the possibility of reciprocal exchanges.

As a working hypothesis we may assume, together with HERSHEY and ROTMAN (1949) that unlinked determinants may be located in different reactivation units, possibly transferred by a gene pool mechanism, while linked determinants may be located in the same reactivation unit. If reciprocal exchanges were found to occur, then homologous units or groups of units should be supposed to pair or group together at some stage in the growth process. Techniques recently developed in our laboratory should soon permit a study of the inactivation of individual genetic determinants and a solution of some of these problems. It appears, therefore, advisable to refrain from further discussion at the present time.

The formation of active phage from inactive by transfer of discrete units requires some revision of the interpretation of experiments on irradiation of phage inside infected bacteria with ultraviolet light (LURIA and LATARJET 1947). In case of multiple infection, the survival curves for phage-producing ability immediately after infection indicated suppression by damage of a number of centers, with the sensitivity of individual centers lower than that of extracellular phage particles. It now seems clear that what was measured was the rate of inactivation of individual units rather than of whole particles. The curves for suppression of the phage-producing ability of multiple-infected

bacteria immediately after infection are similar to the curves for the probability of reactivation for a comparable group of irradiated particles. For example, the suppression curve given by LURIA and LATARJET (1947) for bacteria infected by five particles of phage *T2* is very similar to the curve for the probability of reactivation w as a function of r for $x=5$. It is clear that in interpreting experiments on inactivation of intracellular phage it will be necessary to take into account the occurrence of genetic transfers.⁶

SUMMARY

Coli-bacteriophages *T2*, *T4*, *T5*, and *T6* inactivated by ultraviolet light are still adsorbed by sensitive bacteria. Bacteria infected by only one inactive phage particle are not lysed and do not yield active phage. Infection of bacteria with more than one inactive particle leads to lysis and production of active phage in a fraction of the bacteria. This fraction diminishes with increasing doses of radiation and increases with increasing numbers of particles adsorbed per bacterium. The assumption is made that inactivation is due to lethal mutations in a number of genetic "units" of the phage particle, and that production of active phage from inactive is due to recombination of non-lethal units to form active particles. The values of the probability of active phage production calculated from these assumptions agree with the experimental results with certain limitations. In order to explain the very high frequency of recombination, the hypothesis is proposed that phage growth occurs by independent reproduction of each unit followed by reassembly of the units into complete phage particles. The minimum number of units per particle is estimated for various phages.

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⁶ While this paper was in press, one of us (DULBECCO 1949b) discovered that ultraviolet irradiated phages can be reactivated by exposure to visible light of short wave length in presence of bacterial cells. This "photoreactivation" differs from reactivation by multiple infection in many of its features. Photoreactivation does not take place to any appreciable extent under the conditions in which the experiments reported in this paper were performed. A series of experiments of the type exemplified in table 4, but carried out in dim yellow light—under conditions that completely avoid photoreactivation—gave results undistinguishable from those of the earlier experiments, in which no precaution had been taken to control illumination.

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APPENDIX

ON THE RELIABILITY OF THE POISSON DISTRIBUTION AS A DISTRIBUTION OF THE
NUMBER OF PHAGE PARTICLES INFECTING INDIVIDUAL BACTERIA
IN A POPULATION

R. DULBECCO

In calculating the average number \bar{x} of phage particles adsorbed per bacterium (multiplicity of infection) from the number of uninfected bacteria, and, from x , the proportion of bacteria with any given number k of particles, the assumption is made that the distribution of particles per bacterium is a Poisson distribution. One limitation to this assumption may arise from differences in the surface area of individual bacteria. This limitation was analyzed as follows.

In a mixture of P particles and B bacteria, each phage particle has a probability $p = c(1/B)$ to be adsorbed by a given bacterial cell. In actual cases where P and B are large and c is between 0.4 and 0.9, we have: $\bar{x} = cP/B$.

If c is constant for all bacteria, the distribution of k is a Poisson distribution; if not, the distribution of k will be different. We assume, in first approximation, that the adsorption capacity of a bacterium is proportional to its surface, and that the surface is proportional to the length—considering bacteria as cylinders with uniform diameter and negligible end surfaces.

The distribution of bacterial lengths was obtained experimentally on standard cultures of *E. coli* B containing 10^8 cells per ml. Negative stains with

nigrosin were made without fixation, and, after the slides were dried, 764 cells were measured with an ocular micrometer (Filar). The distribution of lengths is given in figure 8, and will be referred to as the "B distribution." Measurements on five cultures gave similar distributions.

For the purpose of obtaining the distribution of phage particles adsorbed per bacterial cell, or "P distribution," we shall split the bacterial population in-

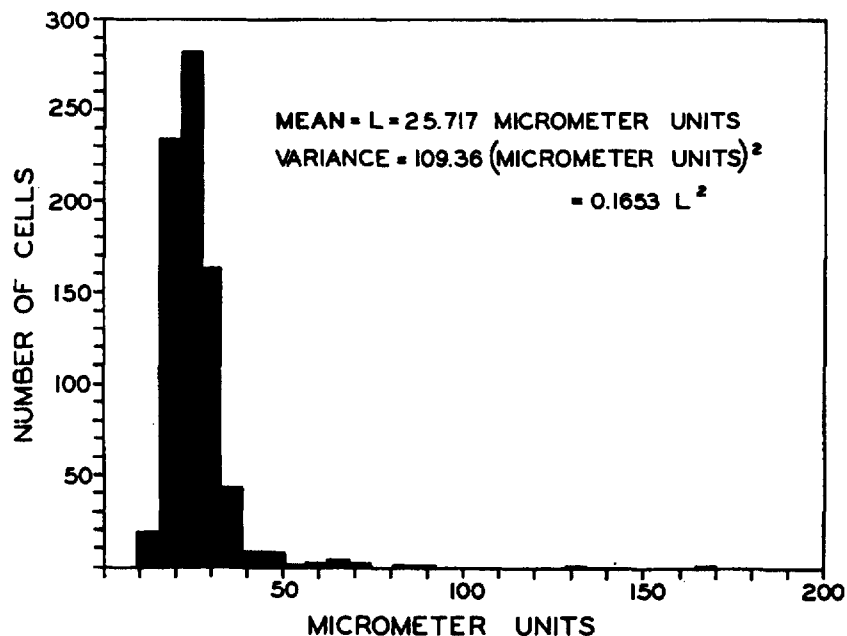


FIGURE 8.—The distribution of bacterial lengths in standard cultures of *Escherichia coli* strain B containing 10^8 cells per ml.

to a number of subpopulations, each comprehending one of the length classes in figure 8, and assume that the adsorption capacity of all cells within each subpopulation is constant.

By the calculation given at the end of this paper, the following properties of the P distribution are derived:

a) its arithmetic mean is equal to x , that is, to the multiplicity of infection, as for a Poisson distribution.

b) its variance is equal to the variance x of the Poisson distribution, plus a term equal to the variance of the B distribution: $\text{var}(P) = x + \text{var}(B)$.

If we express the variance in terms of the mean x taken as the unit of measure, we obtain for our experimental cultures: $\text{var}(P) = x + 0.1653x^2$. This equation expresses the relation between the variance of the P distribution for an ideal population with uniform capacity of adsorption and the variance for a real population with a capacity of adsorption distributed with a given variance.

The P distributions for several values of x , calculated from the actual B distribution, are given in table 8, together with the Poisson distributions

for the same values of x . It is clear that for low multiplicities of infection there is little difference between actual and Poisson distributions. For higher multiplicities, the discrepancies increase; still, for $x=1.5$, they only affect relevantly the proportions of bacteria with 5 or more particles.

TABLE 8

The P distribution for various values of x

"P" columns give the frequencies in the P distribution, "Poisson" columns give the frequencies in the Poisson distribution. All frequencies are multiplied by 10^4 .

K	$x=0.2$		$x=1.5$		$x=2.5$		$x=5$		$x=7.5$		$x=10$	
	P	POISSON	P	POISSON	P	POISSON	P	POISSON	P	POISSON	P	POISSON
0	82115	81873	24686	22313	10400	8208	1409	674	228	55	32	5
1	15977	16374	32918	33468							218	45
2	1735	1637	23197	25101							765	227
3	155	109	11658	12551							1908	757
4	14	6	4751	4707							3739	1892
5	2		1721	1412							6065	3784
6			611	353							8411	6303
7			238	76							10218	9009
8			109	14							11081	11261
9			62	2							10924	12512
10			38								9942	12512
11			29								8489	11375
12			19								6902	9479
13			12								5383	7292
14			8								4089	5208
15			5								3036	3472
16			3								2219	2170
17			1								1600	1276
18			1								1140	709
19											810	373
20											579	187
21-23											931	147
24-26											464	11
27-30											293	
31-33											172	
34-36											110	
37-39											67	
>39											305	

The calculation of x from the bacterial survival—assuming (B uninfected)/ $B=e^{-x}$ —only gives reliable results for values of x up to about 2. For higher multiplicities, it leads to an underestimation of x .

THEORY

The bacterial population is considered as a mixture of an infinite number of subpopulations homogeneous in adsorption capacity. Within each subpopulation there is a Poisson distribution of phage particles per bacterium, whose frequency function is:

$$f_j(k) = \frac{e^{-x_j} x_j^k}{k!} \quad (1)$$

where x_j is the multiplicity of infection in the j th subpopulation. x_j is obtained from the B distribution of bacterial lengths: if we call l_j the length of the bacteria in the j th class, we have:

$$x_j = x \frac{l_j}{L} \quad (2)$$

where x = multiplicity of infection in the total population, L = arithmetic mean of the B distribution (average bacterial length).

The frequency function of the distribution of phage particles per bacterium in the *total* population (P distribution) is

$$f(k) = \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} \frac{n_j e^{-x_j} x_j^k}{k!} \quad (3)$$

where n_j is the frequency of the j th subpopulation in the B distribution

$$\sum_{j=0}^{\infty} n_j = 1$$

The Poisson distribution of phage particles per bacterium within each homogeneous subpopulation of bacteria has the following first two moments:

First moment about the origin = $(\mu_1')_j = x_j$

Second moment about the mean = $(\mu_2)_j = x_j$

For the P distribution in the total population, remembering formula (3), we have a mean

$$(\mu_1')_P = \sum_{k=0}^{\infty} k \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} n_j \sum_{k=0}^{\infty} k f_j(k) = \sum_{j=0}^{\infty} n_j (\mu_1')_j = \sum_{j=0}^{\infty} n_j x_j;$$

from formula (2) we obtain:

$$(\mu_1')_P = x. \quad (4)$$

To find the variance $(\mu_2)_P$ of the P distribution, let us remember that, in general,

$$\mu_2 = \mu_2' - (\mu_1')^2 \quad (5)$$

where μ_2' is the second moment about the origin. We have then:

$$(\mu_2')_P = \sum_{k=0}^{\infty} k^2 \sum_{j=0}^{\infty} n_j f_j(k) = \sum_{j=0}^{\infty} n_j \sum_{k=0}^{\infty} k^2 f_j(k) = \sum_{j=0}^{\infty} n_j (\mu_2')_j$$

where $(\mu_2')_j$ is the second moment about the origin of the distribution of phage particles per bacterium in the j th subpopulation. But $(\mu_2')_j = x_j + (x_j)^2$ (since, in general, $\mu_2' = \mu_2 + (\mu_1')^2$), and, for each homogeneous subpopulation, $(\mu_2)_j = (\mu_1')_j = x_j$. We obtain, therefore:

$$(\mu_2')_P = \sum_{j=0}^{\infty} n_j (\mu_2')_j = \sum_{j=0}^{\infty} n_j x_j + \sum_{j=0}^{\infty} n_j (x_j)^2. \quad (6)$$

Finally, introducing the values obtained from (4) and (6) in formula (5):

$$(\mu_2)_P = \sum_{j=0}^{\infty} n_j x_j + \sum_{j=0}^{\infty} n_j (x_j)^2 - x^2. \quad (7)$$

The first term on the right side of equation (7) equals x ; the other two terms equal the variance of the B distribution, expressed as function of x . We obtain therefore,

$$(\mu_2)_P = x + \text{var (B)}.$$